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TECHNICAL MANUSCRIPT 213

SUSCEPTIBILITY OF WHITE CARNEAU PIGEONS TO RESPIRATORY INFECTION BY VEE VIRUS

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SUSCEPTIBILITY OF WHITE CARNEAU PIGEONS TO RESPIRATORY INFECTION BY VEE VIRUS

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In conducting the research reported here, the investigator adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

ABSTRACT

White Carneau pigeons were found to be susceptible to respiratory infection by Venezuelan equine encephalitis virus. With doses as low as 374 mouse intracerebral LD_{50} units inhaled, seven of eight birds exhibited viremias that approached 10^6 $MICLD_{50}$ units per ml of blood. Viremias generally persisted through the third day after exposure. Birds were not obviously ill and showed serologic evidence of immunizing infections.

I. INTRODUCTION

It has been found that certain arboviruses may be transmitted among avian hosts by modes other than mosquite bite. Holden[1] demonstrated contact transmission of eastern equine encephalitis (EEE) virus among penned pheasants. He postulated that infections resulted from ingestion of infected materials or by inhalation of infective droplets. Chamberlain et al.[2] observed contact infections among chicks with the viruses of western equine encephalitis (WEE) and EEE. He concluded that infected droppings were a source of virus. Bourke[3] postulated that ingestion of infective oral secretions accounted for the infections he observed with WEE virus in chicks. These studies strongly suggest that infections occur without insect vectors, but the portals of entry are not certain. There is a need for a quantitative study of respiratory infectivity of arboviruses for birds. It is the purpose of this report to present the results of such experiments with White Carneau pigeons exposed to controlled aerosols of Venezuelan equine encephalitis (VEE) virus. It is believed that definitive data on respiratory infectivity will be of interpretive value in epidemiologic studies.

II. MATERIALS AND METHODS

A. VIRUS

The Trinidad strain of VEE virus was passed 13 times through chick embryos following isolation from donkey brain. The final select egg embryo harvest was clarified by centrifugation and treated with 1000 units/ml penicillin, 100 µgm/ml dihydrostreptomycin, and 200 µgm/ml Achromycin. The same virus product was employed for all bird tests and for serum neutralization studies.

B. BIRD SPECIES

White Carneau pigeons[4] were obtained from Palmetto Pigeon Plant, Sumter, South Carolina. At the time of receipt the birds were about 6 weeks old and had a mean weight of 494 grams. Birds were held in isolation for 1 to 3 weeks before testing began. Throughout holding the birds were fed mixed grain and water freely.

C. VIREMIA AND VIRUS NEUTRALIZATION ASSAYS

Viremias in birds were determined by mouse inoculation. One-tenth milliliter of blood was taken from the alar vein and diluted in 9.9 ml of phosphate buffered saline (PBS) solution containing 100 units per ml of penicillin and 100 µgm per ml of streptomycin. Eight Swiss-Webster strain mice (8 to 12 grams) were inoculated by the intracerebral route with 0.03 ml from each appropriate dilution. Mouse deaths occurring over the period of 24 through 240 hours after injection were ascribed to VEE virus infection. Titers were computed by probit analysis.[5] Virus neutralization tests with bird sera were also conducted by mouse assays, using equal volumes of serum (diluted 1:10 in PBS and inactivated) and virus with the latter diluted serially in tenfold increments. Following incubation of mixtures at 37 C for 1 hour and at 4 C for 2 hours, 0.25 milliliter was injected by the intraperitoneal route. Control virus titrations were conducted similarly except for the use of normal chicken serum. Neutralization results are reported as the difference between titers with normal chicken sera and titers with pigeon sera. Pigeon sera were harvested for neutralization tests before testing began and again three weeks after birds were exposed to viral aerosols.

D. AEROSOL METHODS

The aerosol chamber, agent disseminating device, samplers, and animal exposure and holding facilities were described previously.[6]

A single aerosol was produced in each experiment to expose birds to various dose levels of virus. Graded levels were achieved by permitting the viral aerosol to undergo natural biological decay. Aerosol samples were obtained at each exposure period in quadruplicate and each was assessed separately in duplicate to insure a relatively precise estimate of viral concentration. Virus samples were collected in liquid impingers containing Sorenson's buffer with 20% egg yolk. Samples were diluted in beef heart infusion broth (Difco) with 100 units/ml of penicillin and 100 µgm/ml of streptomycin. Eight mice were injected intracerebrally with 0.03 ml from each dilution. Responses were measured as described for viremias.

E. RESPIRATORY VOLUMES

The breathing rates for estimating inhaled doses of virus were computed from body weights by Guyton's general equation[7] derived from data with mammals. The mean volume was 220 ml/minute. Few references are available that present data obtained with birds, but one that appears to give comparable results[8] indicated a breathing rate for adult pigeons of 132 ml/minute. The general mammalian equation thus provides estimates not greatly different from actual observations on birds but permits adjustments for body weight when other species are tested.

III. RESULTS

In the first experiment, groups of eight pigeons were exposed to graded doses of 1960, 374, and 75 mouse intracerebral LD_{50} ($MICLD_{50}$) units inhaled. Following exposure, the birds were housed in ventilated animal holding cabinets[8] with dosage groups segregated. To test for cross infection, two control birds were housed with each group. During the exposure process there was a possibility of uncontrolled virus in the vicinity of the aerosol vessel. Therefore, groups of three birds accompanied each dosage group, and received identical handling except for exposure. These birds were subsequently housed individually. A final group of six pigeons, housed in a separate cabinet, served as environmental controls.

All 45 birds were bled on the 2nd, 3rd, 4th, and 5th days after the aerosol exposures for tests for viremia.

The results obtained with exposed birds are presented in Table 1. Viremias were found in 5 of 8 birds exposed to the high dose and in 7 of 8 birds exposed to the medium dose. None of the birds exposed to the low dose showed viremia, suggesting that the minimal infective dose was between 374 and 75 $MICLD_{50}$ units inhaled. None of the control birds exhibited viremias on any test day. Three birds died within three weeks after exposure. Two of these were from the high-dose group and one was from the medium-dose group. Of the former, one exhibited a bacterial meningitis. No obvious causes of death were apparent in the other two cases. None of the three showed histopathologic signs of encephalitis.

Results of serum neutralization tests obtained with sera collected before exposures and again 3 weeks later are presented in Table 2. It was considered necessary for at least a 1.0 log increase in virus neutralization to occur to be accepted as a positive serologic response. On this basis, 4 of 5 of the viremic birds from the high-dose group and 6 of 6 of the viremic birds from the medium-dose group were positive. None of the sera from the low-dose group, or from the control birds, showed significant changes in neutralization ability.

To test for possible recrudescense of virus in the blood subsequent to the 5 test days employed above, a 2nd series of pigeon exposures was made. Birds were tested for viremias through 15 days after exposure to respiratory doses of 2291 and 135 $MICLD_{50}$ units inhaled. The virus titers obtained with the high-dose birds are shown in Table 3. In no instance could virus be detected in the blood beyond day 4. Serologically, most of the birds exhibiting viremias also showed significant increases in virus neutralizing antibodies. Surprisingly, there were significant increases in the ability of serum from birds 96 and 99 to

TABLE 1. VIREMIC RESPONSES OF WHITE CARNEAU PIGEONS EXPOSED TO AEROSOLS OF VEE VIRUS

Inhaled Dose[a] with 95% Confidence Limits, $MICLD_{50}$	Pigeon[b]	Log $MICLD_{50}$/ml Blood			
		Day 2	Day 3	Day 4	Day 5
1960	26	<1.5	5.0	4.0	3.6
(1053-3652)	85	3.9	5.9	3.6	<1.5
	30	4.8	4.0	<1.5	<1.5
	21	4.2	<1.5	<1.5	<1.5
	35	4.0	<1.5	<1.5	<1.5
	25	<1.5	<1.5	<1.5	<1.5
	29	<1.5	<1.5	<1.5	<1.5
	50	<1.5	<1.5	<1.5	<1.5
374	14	<1.5	<1.5	4.9	3.8
(271-516)	49	5.3	5.0	3.5	<1.5
	44	<1.5	4.7	4.1	<1.5
	36	4.8	3.4	<1.5	<1.5
	38	4.8	3.3	<1.5	<1.5
	48	3.9	4.0	<1.5	<1.5
	24	<1.5	3.7	<1.5	<1.5
	33	<1.5	<1.5	<1.5	<1.5
75	31	<1.5	<1.5	<1.5	<1.5
(42-133)	34	<1.5	<1.5	<1.5	<1.5
	40	<1.5	<1.5	<1.5	<1.5
	15	<1.5	<1.5	<1.5	<1.5
	28	<1.5	<1.5	<1.5	<1.5
	84	<1.5	<1.5	<1.5	<1.5
	8	<1.5	<1.5	<1.5	<1.5
	12	<1.5	<1.5	<1.5	<1.5

a. Trial Conditions: 80% relative humidity at 80 F.
b. All control birds were negative in tests for viremia (<1.5 log $MICLD_{50}$/ml) on days 2, 3, 4, and 5.

TABLE 2. SERUM NEUTRALIZATION RESULTS WITH SERA FROM WHITE CARNEAU PIGEONS EXPOSED TO AEROSOLS OF VEE VIRUS

Inhaled Dose with 95% Confidence Limits, $MICLD_{50}$	Pigeon[a/]	Viremia Detected	Serology, Log Units of Virus Neutralized	
			Pre-exposure	Post-exposure
1960	26	+	0.1	0.7
(1053-3652)	85	+	0.3	2.7
	30	+	0.0	3.2
	21	+	0.6	1.6
	35	+	0.3	3.0
	29	-	0.7	1.2
374	49	+	0.2	1.7
(271-516)	44	+	0.1	1.8
	36	+	0.1	2.1
	38	+	0.1	2.6
	48	+	0.6	2.6
	24	+	0.6	1.8
	33	-	0.6	0.6
75	31	-	1.3	0.7
(42-133)	34	-	0.4	0.1
	40	-	1.5	0.3
	15	-	1.2	0.8
	28	-	0.5	0.4
	84	-	0.4	0.5
	8	-	0.6	0.5
	12	-	0.5	0.2

a. Control birds showed no significant changes (<1.0 log unit increase) in serum neutralization against VEE virus.

neutralize virus without evidence of having had a viremic infection. In these birds it is possible that either a transient viremia not detectable by the schedule of bleeding was present, or that less than 1.5 log units of virus were present, a level that would not be detectable by the method of titration used. At the low dose neither viremias nor positive serologic responses were obtained. It should be noted that control pigeons employed in the second test were again negative for viremias and showed no serologic responses. Controls were of the same types as indicated for the first test.

The over-all dose-response data are presented in Table 4 along with average viral concentrations in the blood on each day following exposure. Only those birds exhibiting viremias were considered in the averages.

TABLE 3. VIREMIC AND SEROLOGIC RESPONSES OF WHITE CARNEAU PIGEONS EXPOSED TO VEE VIRUS AEROSOLS

Inhaled Dose[a] With 95% Conf. Limits, ($MICLD_{50}$)	Pigeon[b]	Viremia, Log $MICLD_{50}$/ml Blood				Serology, Log Units of Virus Neutralized	
		Day 2	Day 3	Day 4	Days 5-15	Pre-exposure	Post-exposure
2291 (1096-4736)	66	5.4	4.9	3.2	<1.5	0.9	2.2
	75	4.2	>5.5	5.0	<1.5	0.5	3.0
	74	4.6	<1.5	<1.5	<1.5	0.4	3.5
	78	4.3	<1.5	<1.5	<1.5	0.6	3.5
	19	4.0	<1.5	<1.5	<1.5	0.8	3.5
	72	3.2	<1.5	<1.5	<1.5	-0.1	2.7
	96	<1.5	<1.5	<1.5	<1.5	0.5	3.0
	99	<1.5	<1.5	<1.5	<1.5	-0.2	3.4
	100	<1.5	<1.5	<1.5	<1.5	0.3	0.9

a. Trial Conditions: 80 per cent relative humidity at 80 F.
b. All birds receiving inhaled dose of 135 $MICLD_{50}$ and all control birds were negative in tests for viremias (<1.5 log $MICLD_{50}$/ml) and in serologic tests (<1 log increase).

TABLE 4. LEVELS OF VIREMIA AS FUNCTION OF DOSE IN WHITE CARNEAU PIGEONS EXPOSED TO AEROSOLS OF VEE VIRUS

Dose, $MICLD_{50}$ Inhaled	Response, Viremic/ Total Tested	Mean Viremia, Log $MICLD_{50}$'s/ml[a]							
		Day 2		Day 3		Day 4		Day 5	
2291	6/9	4.3	(6)	5.2	(2)	4.1	(2)	<1.5	(9)
1960	6/8	3.4	(4)	4.0	(3)	2.5	(2)	3.6	(1)
374	7/8	4.7	(4)	4.0	(6)	4.2	(3)	3.8	(1)
135	0/9	<1.5	(9)	<1.5	(9)	<1.5	(9)	<1.5	(9)
75	0/8	<1.5	(8)	<1.5	(8)	<1.5	(8)	<1.5	(8)

a. Numbers in parentheses indicate the number of birds showing viremias and entering into computation of the average viremia.

IV. DISCUSSION

White Carneau pigeons were shown to be highly susceptible to Venezuelan equine encephalitis virus by the respiratory route. The infections resulted in viremias that in some birds exceeded five log units of mouse $ICLD_{50}$ per ml of blood and persisted up to four days after exposure. In no instance did virus reoccur in the blood over the next eleven days. There was no apparent effect of dose level on viremic patterns after a viremic-inducing dose was reached. There was good serologic response to infections. In general, viremia and the subsequent appearance of serum neutralizing antibodies correlated well.

The infections did not appear to be severe, as evidenced by the general healthy appearance of birds throughout the experiments. The three deaths that did occur were considered to be due to causes other than VEE virus infection.

The results suggest the possibility of arbovirus transmission from bird to bird by the respiratory route. A number of instances have been reported[3] of WEE and EEE viruses in the feces and in the oral secretions of infected birds. It is possible that VEE virus may also be excreted. The final step of the cycle requires only aerosolization of excreted virus in sufficient concentrations for respiratory infections, a condition that might be realized in densely populated flocks or with nesting.

It was of interest to compare the results obtained by respiratory exposure with those provided by subcutaneous (sc) injection of virus. Chamberlain[3] conducted a study with VEE virus in which pigeons were injected sc with 346 and 2000 mouse intraperitoneal (MIP) LD_{50} units. It is hazardous to relate doses and viremic responses based on MIP assay, to those of this study, based on MIC assay, quantitatively. Although it has been possible in this laboratory to establish a titer relationship of MIC/MIP (5:1) within one harvest of our Trinidad strain, it has also been found that passage history affects the relationship.[15] However, some similarities between responses are suggested. Chamberlain found that viremias persisted through 66 hours after infection with the level apparently independent of his dosage. In our studies, viremic levels were similar for the inhaled doses and, in the majority of cases, persisted for about the same period.

Chamberlain reported a mean of 2.4 log units of virus neutralized by sera following viremias from sc injections of 2000 and 346 $MIPLD_{50}$ units. In our test with similar assay procedures, the mean viral neutralization in logarithms was 2.5, three weeks after viremic responses to inhaled doses of 2291, 1960, and 374 $MICLD_{50}$ units. Obviously there were at least no major differences between the respiratory and sc routes in terms of serologic response. Finally, it should be noted that in both studies there were no obvious outward signs of infection in the avian hosts.

The cagemates employed in the first experiment with the high-dose birds showed no evidence of infection as measured by viremia and by serum neutralization test. This may have indicated that bird-to-bird infections do not occur, at least with the host-virus combination tested, or that conditions were not suitable for such transmission. With respect to the latter, the holding area for the eight infected and two control cagemates was 36 square feet. The abundant ventilated space may have prevented adquate association for infections to occur.

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